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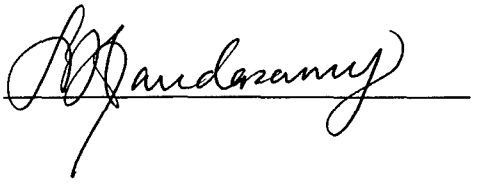

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The goal of this proposal is to test the hypothesis that loss of expression of a novel protein contributes to tumor growth, invasion and metastases, and its expression suppresses these biological events. We have identified, isolated and partially characterized a 55 kDa nuclear matrix protein from human breast tumor cells (hence forth referred to as nmt55). This novel protein is expressed in some estrogen receptor positive (ER+) tumors but was completely absent in ER- tumors. Loss of expression of this novel protein correlated strongly with tumor size ($p<0.03$) and loss of ER and PR ($p<0.001$). As the tumor size increased, the expression of nmt55 was not detected at the protein level. Because increased tumor size is associated with metastases, we postulate that loss of nmt55 expression is associated with molecular and cellular changes linked to cellular differentiation leading to loss of ER expression, and development of hormone-independent tumor growth, invasion and metastases. We have cloned the cDNA for nmt55 and generated site-directed polyclonal antibodies. We are currently investigating the function of nmt55 using biochemical and molecular biology approaches. The information derived from these studies will help determine the potential role of this novel nuclear matrix protein (nmt55) as a marker of tumor progression and metastases. These studies may provide critical information needed for early detection of potentially metastatic tumors, and improve diagnosis, prognosis and in developing strategies for therapeutic management and care of breast cancer patients.

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Matthew Pavao 7-30-99

PI - Signature Date

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# Biochemical and Molecular Characterization of a Novel Nuclear Matrix Protein in Human Breast Cancer: Relationship to Tumor Hormonal Status

## Introduction:

The development of breast cancer is thought to be a multi-stage process (1). The progression of this disease is associated with cellular and molecular changes. Thus, initiation and progression may be related to loss of chromosomal material and ultimately specific gene function (s). Some of these cellular and molecular changes may be accompanied with tumor cell acquisition of metastatic potential. There is an urgent need for identification of node-negative patients whose tumors have metastatic potential. Several tumor markers have been used in assessing tumor changes linked to poor prognosis. These include loss of ER and PR expression (2), high blood vessel count (angiogenesis) (3), amplification of *erbB2/HER2/neu* gene (4) and decreased activity of *nm23* gene (5). None of these markers alone, however, predict with complete reliability which node-negative patients will likely relapse. We have found that primary human breast tumors express a 55 kDa nuclear protein, which is absent in estrogen receptor negative tumors (6). This observation suggested that this protein may be related to tumor hormonal status and may represent a useful tumor marker. We have carried out preliminary studies to characterize this nuclear protein (referred to as *nmt55*) from human breast tumors and MCF-7 cell line, using site-directed monoclonal antibodies and polyclonal antibodies. In this research, we have undertaken biochemical and molecular biology approaches to investigate the functions of this protein and its potential role in regulation of human breast cancer cell growth.

## Results:

### A. Studies on Association of nmt55 with RNA Binding Proteins

Based on the observed strong association between nmt55 expression and ER and PR status in primary human breast tumors (6), we undertook molecular and biochemical approaches to evaluate the potential biological function of nmt55 in regulating cell growth. The predicted nmt55 amino acid sequence suggested that nmt55 contains a bipartite RNA binding domain and posses strong homology to RNA binding proteins (9-11). This suggested that nmt55 may interact with other potential RNA binding proteins and may be involved in RNA processing. To test this premise, we carried out experiments in which MCF 7 cells were labeled, *in situ*, with $^{35}S$ methionine, extracted and the total extracts were immunoprecipitated with monoclonal and polyclonal antibodies, directed to specific domains of nmt55. Incubation of total cellular extracts with monoclonal antibody NMT1 (raised against the carboxyl terminus of nmt55), polyclonal antibody NMT5 (raised against a unique peptide in the carboxyl terminus of nmt55) or polyclonal antibody NMT4 (raised against a unique peptide in the amino terminus of nmt55) resulted in immunoprecipitation of labeled protein bands at 100 and 55 kDa, respectively. In contrast, incubation of cellular extract with pre-immune serum or unrelated monoclonal antibody (ER-213), raised against ER, did not result in immunoprecipitation of these specific proteins. These results suggest the specific interaction of nmt55 with a 100 kDa protein. Literature screening of RNA binding proteins indicated a host of proteins with molecular mass ranging from 35 - 100 kDa (9-11). The 100 kDa band may represent the well characterized polypyrimidine tract binding protein/splicing factor (PSF) (9).

To confirm this finding, MCF 7 nuclear extracts were immunoprecipitated with monoclonal antibody NMT1 to immunoprecipitate nmt55 and associated proteins. The precipitates were electrophoresed and immunoblotted using specific antibodies to either nmt55 or polypyrimidine tract binding protein/splicing factor (PSF). Antibodies raised against PSF detected a specific 100 kDa band in the co-immunoprecipitate suggesting that nmt55 is associated with PSF. To further substantiate this observation, we carried out immunoprecipitation of MCF 7 nuclear extracts with antibodies raised against PSF and immunoblotted with antibodies raised against nmt55. The data obtained showed that antibodies raised against nmt55 protein immunodetected nmt55 in the co-immunoprecipitate. These observations indicate that

nmt55 associates with polypyrimidine tract binding protein/splicing factor (PSF) and that nmt55 may play an important role in regulation of RNA processing and cellular function.

## B. Interactions of nmt55 with DNA

Examination of the protein primary structure suggested that nmt55 may contain a helix-turn-helix domain which may be critical to binding to DNA (6). Although it is not known if this protein binds to DNA, p54nrb, a protein with a high degree of homology to nmt55, cloned from HeLa cells, was shown to bind to a specific DNA sequence derived from the murine long terminal repeat intracisternal A-particle proximal enhancer element (IPE) (10,12). To investigate potential binding of nmt55 to DNA, we used gel mobility shift assays to determine the putative DNA binding elements for this protein. Using homology sequences with other nuclear proteins, we have shown that nmt55 binds to intracisternal A-particle proximal enhancer element (IPE), with specific DNA sequence, ATCATCAGGGAGTGACACGTCCGA. nmt55 bound IPE probe specifically since unlabeled IPE competed for binding of proteins to IPE probe. To further determine that this IPE probe represent a binding site for nmt55 and further characterize sequence requirements for binding, interaction of nmt55 with IPE probe was carried out in the presence of mutated sequences of IPE and with DNA representing the cAMP response elements (CRE). Mutated IPE (mt2), GATCATCAGGGAAATTTACGTCCGA, reduced nmt55 binding to IPE probe. Mutated IPE (mt3), GATCATCAGTTTGTGACACGTCCGA, and CRE, GATCTTCCCCGTGACGTCAACTCGGC, did not compete for IPE probe binding to nmt55. Mutated IPE (mt4), GATCATCAGGGAGTGTTGCGTCCGA, was very effective in displacing IPE probe from nmt55.

Another protein with a high degree of homology to nmt55, murine Non O, has been shown to bind to a specific DNA sequence (Oct 2) (11). These octomeric motifs have been identified in the promoter and enhancer regions of many genes (11). We were unable to demonstrate binding of nmt55 with the Oct 2 sequence utilizing the gel mobility shift assay approach. The native (endogenous) cellular DNA sequences, which bind nmt55 are unknown at present. These data indicate that nmt55 binds specific IPE DNA sequence but does not bind the Oct 2 DNA sequence and may play a role in gene regulation.

## C. Interactions of nmt55 with RNA

Further examination of the protein primary structure suggested that nmt55 contains a bipartite RNA binding domain which is critical for binding to RNA (6). Although it is not known if this protein binds to RNA, its high degree of homology to other RNA binding proteins ($p54^{nrb}$) (10) and its association with PSF suggests it may bind to RNA. To investigate possible binding of nmt55 to RNA, MCF 7 cells were permeablized and then treated with RNase or DNase to solubilize nmt55. The cells were then washed, and the nmt55 was detected with NMT5 antibodies using immunofluorescent conjugated-secondary antibodies. Control experiments utilized fluorescentlabeled antibody raised against a nuclear vault protein. DNase treatment did not alter the immunoflouresecne pattern compared to control. In contrast, RNase treatment solubilized nmt55 but not the vault protein as shown by loss of immunofluorescence with nmt55 antibodies. These data indicate binding of nmt55 to RNA, *in situ*.

## D. Construction of Sense and Antisense Probes for Transfection into Breast Cancer Cell Lines

To investigate the possible role of nmt55 *in situ*, breast cancer cell lines will be stably transfected with sense and antisense cDNA construct for nmt55, or the vector alone. We have chosen the pCIneo vector (Promega) since it is suitable for eukaryotic expression. This vector has been used successfully for high-level expression because it has the enhancer-promoter sequences from the immediate early genes of the human cytomegalovirus (CMV) which have been further optimized for high-level transcription (7). This vector also includes G418 (neomycin) resistance for clonal selection. Two sense constructs of nmt55 have been prepared. A 1.6 kb XbaI/EcoRI restriction fragment was subcloned into NheI/EcoRI digested pCIneo. This construct contains 115 bp of 5' untranslated, the entire coding sequence and 64bp of 3' untranslated sequence. The full length 2.5 kb XbaI/XhoI cDNA fragment was subcloned into NheI/XhoI digested pCIneo. These plasmids have been prepared and are currently being transfected into the MCF 7 cell line. Transfectants are being grown in the presence of G418 (neomycin) to select positive stably integrated clones. Expression of nmt55 mRNA and protein in the selected clones will be determined by Northern and Western blots of nuclear KCl-extracts, respectively. It should be noted that the 1.6 kb nmt55 construct is expected to express a unique transcript, further facilitating selection. The number of integrative plasmids will be estimated by Southern blots of genomic DNA derived from the clonal cell lines.

Antisense cDNA plasmid constructs have been prepared by subcloning the 459bp SacI/EcoRI nmt55 fragment into EcoRI/SmaI digested pCIneo. For this purpose, the SacI site was filled-in using T4-DNA polymerase. This construct contains the 3' coding region and 64 bp of 3' untranslated sequence, and in this reverse orientation, is expected to make antisense RNA driven by the CMV promoter (7,8). The coding region represented here does not include the predicted RNA binding domain, the Oct-2 -like helix-turn-helix, or the acidic/basic region, which is homologous to putative RNA binding proteins and transcription factors. This should circumvent possible cross-hybridization with other mRNAs, leading to undesirable pleotrophic outcomes. A second construct was made, which included all the 3' untranslated sequence in the reverse orientation 1.5 kb T4-DNA polymerase filled in SacI/XhoI restriction fragment into XhoI/SmaI digested pCIneo. Both constructs are currently being tested (transfection analyses) in MCF-7 cells for their ability to "knock-out" (reduce) nmt55 protein expression, as determined by Western blot analysis. Northern blot analysis will be performed to establish the loss of nmt55 mRNA. The number of integrative plasmids will be estimated by Southern blots of genomic DNA derived from the clonal cell lines.

## Summary and significance of the studies

In these studies we have demonstrated that nmt55 interacts with RNA processing protein PSF and is capable of binding to specific and unique DNA sequences. We further demonstrated that nmt55 interacts with RNA, *in situ*. These observations, together with its ability to interact with PSF, suggests that nmt55 may play an important role in RNA metabolism and/or processing. We have developed sense and antisense constructs to test nmt55 function in tumor cell lines and relate its expression to the presence of estrogen receptor. Also, we are currently developing GST fusion proteins to further dissect the role of nmt55 functional domain interaction with RNA and RNA binding proteins. The association of nmt55 expression with tumor hormonal status (ER and PR) in human breasts tumors and its putative function as an RNA binding protein suggests a key role in cellular growth and function. The data obtained suggest that nmt55 binds to RNA and RNA processing proteins and may have an important role in regulation of RNA metabolism. This may be critical in tumor cell growth and tumor progression.

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Protein p54nrb/NonO. Molecular and Cellular Biology. 17 (2): 677-686, 1997.

# Appendix

## Key Research Accomplishments

- Demonstration of interaction between nmt55 and polypyrimidine tract binding protein/splicing factor (PSF). Determined by co-immunoprecipitation experiments.

- Demonstration of nmt55 binding to DNA. Determined by Gel Mobility Shift Assay experiments.

- Demonstration of nmt55 binding to RNA. Determined by Solubility experiments.

- Development and Construction of specific sense and antisense probes to be used for transfection studies.

## Reportable Outcomes

At present, there are no reportable outcomes.

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